Collections 16 (D6)

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12P 17/04, C12N 1/12
// (C12N 1/12, C12R 1:89)
(C12P 17/04, C12R 1:89)

A1

(11) International Publication Number: **WO 93/19192**

(43) International Publication Date: 30 September 1993 (30.09.93)

(21) International Application Number: PCT/US93/02429

(22) International Filing Date: 18 March 1993 (18.03.93)

(30) Priority data:
07/853,379 18 March 1992 (18.03.92) US

(71) Applicant: BIO-TECHNICAL RESOURCES [US/US]; 1035 South Seventh Street, Manitowoc, WI 54220 (US).

(72) Inventors: HUSS, Ronald, John ; RUNNING, Jeffrey, A. ; SKATRUD, Thomas, J. ; 1035 South Seventh Street, Manitowoc, WI 54220 (US).

(74) Agents: WINSLOW, Don, O. et al.; E.I. du Pont de Nemours and Company, Legal/Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, KZ, LK, MG, MN, MW, NO, NZ, RO, RU, SD, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: L-ASCORBIC ACID PRODUCTION IN MICROORGANISMS

(57) Abstract

Improved heterotrophic biosynthetic production of ascorbic acid is obtained using ascorbic acid-producing microalgae in particular of the genus *Chlorella*, as the microorganism source and growing, the culture under a controlled pattern of carbon source supply. Greatly improved ratios of ascorbic acid to total carbon source supplied as well as enhanced concentrations of ascorbic acid in the fermentor are obtained. *C.pyrenoidosa* UV 101-158 was deposited at the A.T.C.C. on June 27, 1985 and given Accession No. 53170.

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TITLE
L-ASCORBIC ACID PRODUCTION IN MICROORGANISMS

CROSS-REFERENCES TO RELATED APPLICATIONS

This case is a continuation-in-part of U.S. Application Serial Number 07/650,886, filed March 5, 1991, which is a continuation application of U.S. Patent 5,001,059, filed July 1, 1985 and issued March 19, 1991.

BACKGROUND OF THE INVENTION

1. Field of the Invention:

This invention relates to a heterotrophic process for the improved production of L-ascorbic acid by microorganisms, in particular microalgae, in nutrient media containing a suitable carbon source. In particular, the invention relates to such process which produces high concentrations of L-ascorbic acid, preferably high concentrations per unit weight of cell mass. The invention also provides new mutagenized microalgae species suitable for use in the invention L-ascorbic acid process.

2. Description of Related Art:

L-ascorbic acid is an important nutrient supplement, which finds wide application, in vitamin capsules and as a nutrient supplement in foods for both humans and other Vitamin C requiring animals. L-ascorbic acid is a bulk chemical which is highly price sensitive and requires economic and efficient production to be marketable. Therefore, there is substantial interest in being able to develop processes employing microorganisms which provide for efficient conversion of nutrients resulting in efficient production of L-ascorbic acid.

Loewus, F.A., in L-Ascorbic Acid: Metabolism, Biosynthesis, Function, The Biochemistry of Plants, Vol. 3, Academic Press, Inc., pp. 77-99, 1980, provides a review of the sources and biosynthesis of L-ascorbic acid. Descriptions of production of ascorbic acid in algae may be found in Vaidya et. al., Science and Culture (1971) 37:383-384; Subbulakshmi et. al., Nutrition

Reports International (1976) 14:581-591; Aaronson et. al., Arch. Microbiol. (1977) 112:57-59; Shigeoka et. al., J. Nutr. Sci. Vitaminol. (1979) 29:29-307; Shigeoka et. al., Agric. Biol. Chem. (1979) 43:2053-2058: Bayanova and Trubachev, Prikladnaya Biokhimiya i Mikrobiologyia (1981) 17:400-407 (UDC 582.26:577.16); and Ciferri, Microbiological Reviews (1983) 47:551-578.

The prior art heterotrophic processes are not entirely satisfactory for commercial use: their utilization of the carbon source for ascorbic acid production is generally poor and the vitamin is produced in undesirably low concentrations.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a process for the heterotrophic biosynthesis of L-ascorbic acid from a carbon source which results in enhanced utilization of the carbon source. It is another object to provide such a process which affords the vitamin in high yields. It is a further object to provide novel high L-ascorbic acid-producing microorganisms as new compositions of matter.

Accordingly, the present invention provides an improved process for L-ascorbic acid production, which process comprises heterotrophically growing cells of an L-ascorbic acid producing microorganism in a nutrient medium containing a carbon source and dissolved oxygen (O_2) in amounts suitable for growth and L-ascorbic acid production, allowing the organism to grow in an initial stage to a high cell density accompanied by intra cellular L-ascorbic acid production and the substantial complete depletion of the carbon source, maintaining the cells in the substantially depleted carbon source state until cell growth substantially ceases and subsequent addition of the carbon source in controlled amounts results in the formation of additional quantities of L-ascorbic acid with substantially little or no increase in cell density, and continuing the controlled carbon source addition until a desired increase in L-ascorbic acid production is attained with substantially little or no increase in cell density.

Thus, improved utilization of the carbon source is observed in relation to L-ascorbic acid (L-AA) production, while obtaining an enhanced yield, as evidenced by an increase in total L-AA expressed as mg L-AA/liter of

solution, and preferably also by an increase in the specific formation of L-AA expressed as mgs of L-AA per gram of dry weight of cells.

The present invention further provides a new high L-AA producing mutagenized microalgae, more specifically: *Chlorella pyrenoidosa* UV101-158 which is derived from *C. pyrenoidosa* isolate UTEX 1663 and has been deposited at the American Type Culture Collection (ATCC) on 6/27/85 and given Accession No. 53170; *Chlorella regularis* UV5-280 derived from C. regularis UTEX 1808; Prototheca zopfii UV3-132 derived from *P. zopii* UTEX 1438; *Ankistrodesmus braunii* UV2-370 derived from *Ankistrodesmus braunii* ATCC 12744. UTEX is the Culture Collection of Algae at The University of Texas at Austin.

The production of these new microalgae compositions of matter and their effectiveness for the production of L-AA in the process of this invention are described more fully below.

DESCRIPTION OF THE PREFFERED EMBODIMENTS

In general, the invention process involves three key stages: (1) an initial cell growth stage wherein a microorganism, preferably a microalgae, capable of producing L-AA heterotrophically is grown heterotrophically in a fermentor containing an effective carbon source at a first concentration and dissolve O_2 each in amounts sufficient for the organism to grow to a high cell density accompanied by the formation of intra cellular L-AA and the substantially complete depletion of the carbon source; (2) a substantially completely depleted carbon source stage wherein the cells of the microorganism are allowed to remain in such depleted carbon source state until cell growth substantially ceases; and (3) a controlled carbon source addition stage wherein the carbon source is fed to and maintained in the fermentor at a second concentration lower than the first concentration and effective to result in the production of additional amounts of L-AA in the presence of disolved O_2 without resulting in a substantial increase in the density of the cells.

The addition of the carbon source at the lower concentration (at which L-AA production is favored over cell growth) can be continued until the ability of the microorganism to produce L-AA is substantially exhausted.

This point can be determined by monitoring the L-AA concentration and cell density with time throughout the process.

Other stages of the process include the harvesting of the cells and the separation and recovery of the L-AA substantially free of cellular material in accordance with procedures known to the art.

The L-AA product recovered fromt the cells (biomass) can be utilized as such. Alternatively, the L-AA ladened biomass itself can be used as a vitamin C enriched animal feed composition or feed supplement including for use in the aquaculture of fish.

The microorganisms for use in this invention may vary widely provided they are L-AA producers, in particular such organisms capable of producing intracellular L-AA heterotrophically. Preferred microorganisms are the L-AA producing green microalgae, especially for reasons of economy the so-called high-producers thereof, sometimes referred to as over-producers. Organisms showing promise as potential high-producers of L-AA can be identified using standard fermentation procedures for cell growth accompanied by L-AA production. They are then preferably mutagenized using physical or chemical mutagenizing means, e.g. U.V. light, x-rays, N-methyl-N'-nitro-N-nitrosoquanidine, dimethyl sulfate or the like agent known to the art. Mutagenized L-AA overproducers produced by such treatments can be advantageously determined with redox dyes. Further, by employing analogs to metabolic intermediates to ascorbic acid or inhibitors of the ascorbic acid synthesis, microorganisms may be selected that are capable of maintaining or increasing L-AA production in the presence of chemical interference.

Preferred progeny of the above procedures are those microorganisms providing improved specific formation of L-AA as measured by milligrams of L-AA per gram of cells (dry weight basis). These progeny may then be further separated into individual clones and further subjected to the above procedures.

One preferred microorganism is of the green microalgae genus *Chlorella* in particular *Chlorella pyrenoidosa* strain, UV101-158, a high L-AA producing mutant descended from strain UTEX 1663, by mutagenizing UV light. *C. pyrenoidosa* UV 101-158 has been deposited with the ATCC and given Accession No. 53170. Another preferred *C. pyrenoidosa* strain is UV232-1, the highest intracellular L-AA produced to date. Other

representative and suitable species of the genus *Chlorella* are: *Chlorella regularis* strain UTEX 1808; and *C. regularis* UV 5-280, a UV generated high L-AA producing mutant of strain UTEX 1808. Still other suitable L-AA producing microalgae also able to grow heterotrophically are those belonging to the genera *Prototheca* and *Ankistrodesmus*. Representative *Prototheca* species are *P. zopfii*, strain UTEX 1438 and *P. zopfii* UV3-132, a UV-generated high L-AA producing mutant of UTEX 1438. Representative *Ankistrodesmus* species are *A. braunii* ATC 12744 and *A. braunii* UV 2-370, a high L-AA producing UV-generated mutant of ATC 12744.

It will be noted, in each above case, the mutagenized offspring is a higher L-AA producer than its parent organism. Further, as shown below, each organism produces a higher maximum concentration of L-AA, in terms of mg L-AA/liter of nutrient medium, under the invention conditions as defined above than under conventional, prior art conditions.

In carrying out the process, a nutrient culture medium is innoculated with an actively growing culture of the microorganism in amounts sufficient to result after a reasonable growth period in a high cell density generally accompanied by a first, generally low concentration of L-AA. Typical initial cell densities are generally in the range of from about 0.15 to 0.4g/L based on the dry weight of the cells. The culture medium includes the carbon source, a variety of salts and generally also trace metals. It also includes a source of molecular O_2, generally air, fed in amounts that are growth-promoting in conjunction with growth-promoting amounts of the carbon source. In other wrds, both an effective carbon source and O_2 must be available to the microorganism to achieve growth to a high cell density.

The carbon source is normally a source of L-galactose or D-glucose, preferably glucose, for reasons of economy. The source of glucose may be any carbhydrate that can be converted in situ to glucose, e.g. molasses, corn syrup, etc. The total amount of glucose source employed can vary broadly depending upon the particular organism and the result desired. Normally, with a high L-AA producing organism such as *C. pyrenoidosa* UV 232-1, the total amount of glucose source employed would, if not metabolized, provide a concentration of about 65 to 90, more usually about 75 to 85, and preferably about 80 g/l calculated as glucose. Usually, about 15 to 30% of the total glucose will be added initially, more usually about 20 to 25% of the total glucose. The glucose is normally added initially and during the fermentation

along with other additives identified below. During the initial glucose source addition and fermentation period, which is the period of unrestricted cell growth wherein the cells are grown to a relatively high cell density, generally accompanied by the formation of L-AA, usually in a relatively low concentration.

The amount of glucose source in the fermentor should be a non-repressing/non-limiting amount, that is, it should optimally promote and not inhibit or unduly limit cell growth. Optimum non-growth limiting concentrations of the glucose source may vary from organism to organism and are readily determined by trial for any particular organism. For example, with *C. pyrenoidosa* UV 101-158 the glucose source concentration is maintained by timely additions in the 15-30 g/l range, found sufficient to promote cell growth while avoiding glucose inhibition of growth.

Desirably, other additives are present initially along with glucose source, and their concentrations in the nutrient medium are generally also continually provided by subsequent additions of the same additives in conjunction with the continued addition of the glucose source. The ratio of the concentrations of these additives to the concentration of the glucose source may be the same or different throughout the fermentation.

Among the additives which may have a different ratio to glucose in the amounts added incrementally as compared to the total amounts added to the fermentor are the alkali metal phosphates, e.g.,sodium and potassium phosphates, particularly as the dibasic sodium phosphate and the monobasic potassium phosphate. The total amount of the dibasic sodium phosphate is typically about 1 to 2 total g/l, usually about 1 to 1.5 total g/L and preferably about 1.3 total g/L. The amount initially present in the fermentor of dibasic sodium phosphate is usually about 35 to 50%, more usually about 40 to 45% of the total amount of dibasic sodium phosphate added. The total amount of monobasic potassium phosphate is usually about 1.5 to 3g/L, more usually about 2 to 2.5g/L. The amount initially present is generally about 40 to 50% of the total amount, more usually about 45 to 50% of the total amount.

In addition to the above additives, a biologically acceptable chelating agent, such as trisodium citrate is advantageously added in a total amount of from about 0.8 to 1.2g/L, usually about 1g/L. Monobasic sodium phosphate generally present in from about 0.8 to lg/L, preferably about 0.95 to lg/L. A biologically acceptable mineral acid is added to maintain the trace metals in

solution and also to neutralize the ammonia that is usually employed as the nitrogen source. Conveniently, conc. sulfuric acid is employed for this purpose in amount of about 1 to 2, more usually about 1.2 to 1.5ml/L. Among the metals, magnesium is present in about 0.1 to 0.2g/L, preferably about 0.1 to 0.15g/L, particularly as a physiologically acceptable salt, e.g., sulfate. The amount of iron and copper employed is limited, since these metals repress ascorbic acid formation. Iron (ferrous) is present initially in from about 5 to 7mg/L, preferably about 5.5 to 6mg/L, and is not included in any subsequent additions. Copper is present in relatively minute amounts, generally from about 1 to 50ug/g of glucose.

The trace metal solution, described below is used in total amount of from about 10 to 15ml/L, more usually about 12 to 14ml/L. Based on glucose, the trace metal solution coprresponds to 0.1 to 0.2ml/g.

Conveniently, a solution is prepared for addition during the course of the fermentation and has the following composition.

TABLE 1

Medium formula

COMPONENT	CONCENTRATION (Relative to glucose)
glucose	1.0
trisodium citrate, dihydrate	0.0125
magnesium sulfate, anhydrous	0.0082
monobasic sodium phosphate	0.0116
monobasic potassium phosphate	0.0238
dibasic sodium phosphate	0.0121
trace metal mixture	0.1675ml/g
sulfuric acid 98% (w/w)	0.0329

Nitrogen supplied is by anhydrous ammonia. This is also used as pH control. Actual N level in media is determined by acidity of media and buffer capacity of media.

The trace metal mixture and solution have the following composition.

TABLE 2

COMPONENT	Trace Metal Solution CONCENTRATION (conc. in stock solution) mg/liter
calcium chloride, dihydrate	3102
mangaanese (II) sulfate, monohydrate	400
copper (II) sulfate, monohydrate	0.4
cobalt (II) chloride, pentahydrate	40
boric acid	160
zinc (II) sulfate, heptahydrate	400
sodium molybdate, dihydrate	19
vanadyl sulfate, dihydrate	20
nickel (II) nitrate, hexahydrate	8
sodium selenite	18

The stock solution of trace metals is prepared by dissolving the appropriate amounts of the various compounds in distilled water containing a trace of HCl so that the final volume is one liter and contains 20ml of concentrated HCl. Distilled water is used to ensure the proper ratio of component trace metals.

While a number of the salts are referred to as mono- or dibasic, it should be understood that this is a matter of convenience and not necessity. These compounds act as buffers and therefore the extent of protonation will vary with the pH of the medium.

In carrying out a fermentation, dibasic sodium phosphate and monobasic sodium phosphate will be dissolved into about 75 to 90% of the total medium, usually about 80 to 90% of the total medium, to be added to the fermentor.

The solution to be added incrementally during the course of the fermentation is prepared by combining the individual components in proper ratios. The glucose is dissolved in from about 75 to 85%, preferably about 80% of the water to be used. The citrate, magnesium and sulfuric acid components are combined in an aqueous medium containing from about 5 to 15%, usually about 10% of the water, while the phosphates are combined in

about 5 to 15%, more usually about 10% of the water to be used, followed by the trace metal solution in about 5 to 15%, more usually about 8 to 10% of the total amount of water to be used. To the fermentor containing a portion of the phosphates is added the ferrous salt and about 20% of the above-prepared glucose-salts concentrate. The addition is aseptic, so as to avoid the introduction of any foreign microorganisms.

The nutrient medium is then brought up to the desired temperature. This may vary with the micro-organism but generally is in the range of about 30 to 40oC, preferably about 35oC and the fermentor inoculated with the inoculant generally to provide from about 0.1 to 0.4g/L initial cell density. A small amount of antifoaming agent may be added during the process of the fermentation.

In the first stage of the fermentation, the cells are grown to a high cell density so as to provide a basis for an eventual high total production L-AA, and cell growth is continued until the carbon source is substantially completely consumed, i.e., its glucose equivalent concentration is normally less than 0.1g/L of solution, i.e., cell-free supernatant. The time required may vary with the organism but usually involves a period of about 35 to 50hr, more usually about 40 to 45hr, with a growth rate of about 0.1 to 0.15hr^{-1}.

The pH of the medium can be controlled within desired limits, generally in the range of about 6.5 to 8.0, by the addition of anhydrous ammonia as needed.

The medium is advantageously agitated and aerated during this growth period. The agitation rate is usually at about 200 to 1000 rpm, while the aeration rate is generally in the range of about 0.2 to 0.6L of air/min., although this can vary with the organism and the other fermentation conditions. Aeration provides molecular oxygen (02) to the medium, which is necessary for cell growth and the production of L-AA. Other sources of 02 can be employed, including undiluted 02 gas and 02 gas diluted with inert gas other than N2. Whatever the source of 02, the dissolved 02 content in the medium should be controlled during the course of the fermentation so as to ensure high cell growth and high intracellular content of L-AA. The O_2 content of the nutrient medium can be monitored by standard methods, convently with an O_2 probe electrode.

The glucose available to the microorganism during this initial growth stage can be monitored in the supernatant, i.e., cell-free component of the

medium, by convenient means, e.g., the glucose oxidase enzyme test, HPLC, or other known method. When the glucose concentration drops, it can be replenished as needed by adding aliquots, e.g., 20% aliquots of the glucose-salts concentrate described above, while ensuring that the total glucose concentration remains below growth repressive levels, which as noted above is generally below about 30g/L. Glucose availability is maintained until a desired high cell density is attained, which for *C. pyrenoidosa* UV101-158, for example, is about 35 to 45g/L, preferably about 40g/L, with cell density calculated on a dry cell weight basis. At this point, the glucose content of the medium is allowed to become substantially completely depleted if it has not already reached this state.

When the desired high cell density is achieved and the glucose content of the medium has become substantially completely consumed, the glucose depleted state is maintained, i.e., the organism is starved for a period of time during which the growth of cells substantially ceases and the cell density reaches a maximum. With cessation of cell growth, L-AA production may also substantially cease. In general, however, the cells can utilize stored starch to maintain cell functions including L-AA production to some small extent.

The substantially depleted glucose and no growth state is maintained until the organism on again being provided with glucose source, but in controlled amounts, begins to produce additional quantities of L-AA with little or no increase in cell density. The starvation period may range from minutes or less to hours or more and typically is from about 1 to 4 hours, more usually 2 to 4 hours. The optimum starvation time and the optimum amount and rate of feeding glucose source to the fermentor can be determined for any particular organism by adding glucose source in small test quantities and monitoring the effect on L-AA content and cell density with time. When the ratio of the increase in L-AA to the increase if any in the cell density is greater than the maximum ratio of L-AA to cell density during the growth period, such enhanced L-AA production state is continued by feeding glucose source, generally in equal time increments, in an amount and at a rate favoring L-AA formation over cell density increase. The optimum amount and feed rate for any particular microorganism can thus be readily determined by trial. Typically, with *C.Pyrenoidosa* UV-101-158 as the

microorganism the feed rate is in the range of about 0.005 to 0.05g glucose/hr/g cells taken as dry weight of cells and the glucose content is maintained below growth promoting concentrations, e.g., below about 0.1g/L of solution.

The subject method affords intracellular L-ascorbic acid in high yields, far higher than those from other naturally occurring sources, such as rose hips. Levels exceeding 3.5% of biomass material can be achieved with levels of 4.0% and higher attainable. The concentration of L-ascorbic acid can exceed 1.45g/L and can be 3.3g/L or greater. Based on the substrate consumed, molar yields are attained that are at least about 0.01.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

Sterilized in a 1L fermentor was 0.6L distilled water, 0.23g dibasic sodium phosphate and 0.27g monobasic potassium phosphate. To the phosphate solution was then aseptically added 11.2mg of ferrous sulfate (heptahydrate) in 5ml distilled water and 20ml of sterile glucose-salts concentrate prepared as follows with Groups of nutrients sterilized individually and combined after cooling:

Group 1

56g glucose, food-grade monohydrate
(anhydrous basis) (80g/L)
in 80ml water

****

Group 2

0.7g trisodium citrate dihydrate (1.0g/L)
magnesium sulfate anhydrous (0.66g/L)
and
1ml sulfuric acid (1.4ml/L in 10ml water

****

Group 3

0.65g monobasic sodium phosphate (0.97g/L)
1.3g monobasic potassium phosphate (1.9g/L)
0.6g dibasic sodium phosphate (0.97g/L)
in 10ml water

Group 4

9.4ml trace metal solution

The temperature was raised to 35°C, agitation begun at about 200rpm. Air was passed through the medium at the rate of 0.2 liters per minute (1pm) and 50ml of *Chlorella pyrenoidosa* UV101-158 at a concentration of about 0.3g cells/L added. After 5 hours the agitation rate was increased to 400 rpm, the air flow to 0.4 1pm. After 16 hrs the agitation rate was raised to 550 rpm, the air flow to 0.6 1pm. The agitation rate was later increased to 700 then 800 rpm as noted in Table 3 while the air flow was steady at 0.6 1pm for the remainder of the run.

The following chart describes the conditions and analytical results for the fermentation.

TABLE 3

Time hr	pH	Cell Density G/L	Ascorbic Acid mg/L	Comments
0	6.9	--		
5	6.6	0.7		400rpm; air 0.4 1pm
16	6.9	3.8		550rpm; air 0.6 1pm
21	7.0	9.5		700rpm; add 20ml
24	6.9	14.2		800rpm; add 20ml*
36				glucose depleted
40	7.1	38.6	538	add 4ml+
45	7.2	38.6	654	
48				add 4ml+
51	7.6	38.1	775	add 2ml+
65	7.7	37.8	966	
68	7.8		1050	add 4ml+
92	7.6	37.2	1292	
101	7.3	36.1	1459**	

* glucose/salts concentrate

\+ 20% glucose. This addition was repeated periodically during the course of the fermentation.

** corresponds to 0.04g L-AA/g cell dry weight or 4% by weight of dry biomass

The method employed for determining L-ascorbic acid is described by Grun and Loewus, Analytical Biochemistry (1983) 130:191-198. The method is an ion-exchange procedure, employing a 7.8 x 300mm organic acid analysis column, HPX-87 (Bio-Rad Laboratories, Richmond, CA). The conditions are: mobile phase, 0.013 M nitric acid, flow 0.8ml/min, pressure 1500 psig, detection, UV 245-254nm. With the above conditions, resolution of L-ascorbic acid and isoascorbic is possible.

To determine the grams of cells per liter, he following procedure is employed. A biomass sample (5ml) is transferred to one weighing pan and 5ml of supernatant transferred to a second weighing pan. The supernatant is centrifuged. The pans are dried in a convection oven (105oC for 3hrs). After cooling in a desiccator, the pan contents are weighed. The grams of cells per liter are determined as: (sample weight- supernatant weight) x 200.

Based on the above results, specific formations based on grams of ascorbic acid per gram of cell are achieved of at least 0.04 and the molar yield defined as moles of L-ascorbic acid formed per mole of glucose consumed is at least 0.01 or higher. In addition, the ascorbic acid concentration can be raised to at least about 1.5g/L.

EXAMPLE 2-10

The procedure of Example 1 was repeated substantially as described using: *Chlorella pyrenoidosa* strain UTEX 1663, strain UV101-158, the UV-generated mutant of strain 1663 described above, and strain UTEX 343; *Chlorella regularis* strain UTEX 1808 and strain UV5-280, a UV-generated mutant of strain 1808, *Prototheca zopfii* strain UTEX 1438 and strain UV3-132, a UV-generated mutant of strain 1438 and *Ankistrodesmus braunii* strain ATCC 12744 and strain UV2-370, a UV-generated mutant of strain 12744.

It should be noted the three microalgae genera, namely *chlorella, Prototheca* and *Ankistrodesmus*, selected to illustrate the invention in these Examples, are widely divergent taxonomically and are considered representative of L-ascorbic acid-producing heterotrophic microalgae.

Each of the above species was grown to approximately 40 g/L cell density (dry weight basis) in a fed batch, one-liter stirred jar fermentor. Nutrient nitrogen was supplied by, and pH controlled by, addition of ammonia. After the cells had exhausted the glucose-based nutrients, they entered a period of 1 hr to 3 hrs without additional glucose, after which they were given 0.3 grams of glucose per gram dry weight of cells every 3 hours

(designated in the Table below as glucose pulsing after growth) until twice-daily analyzes indicated that ascorbic acid synthesis had peaked and was in decline. The results of these runs are given in the following Table alongside "Yes" under the subheading "Glucose pulsing after growth".

Controls runs were conducted with the same strain under the same fed-batch conditions until glucose depletion; however, no additional nutrients were added after glucose depletion. This growth condition is indicated by "No" under "Glucose Pulsing After Growth".

TABLE 4

Example	Micro-organism	Glucose Pulsing After Growth	Maximum Ascorbic Acid Concentration (mg/liter)	Maximum Specific Formation (mg/g cells)
Chlorella pyrenoidosa				
Control	UTEX 1663	No	38	1.07
2	UTEX 1663	Yes	54	1.24
Control	UV101-158	No	570	12.9
3	UV101-158	Yes	753	17.0
Control	UTEX 343	No	38	0.7
4	UTEX 343	Yes	38	0.66
Chlorella regularis				
Control	UTEX 1808	No	15	0.34
5	UTEX 1808	Yes	28	0.38
Control	UV5-280	No	26	0.54
6	UV5-280	Yes	32	0.65
Prototheca zopfii				
Control	UTEX 1438	No	24	0.7
7	UTEX 1438	Yes	56	2.1
Control	UV3-132	No	50	2.5
8	UV3-132	Yes	157	6.8
Ankistrodesmus braunii				
Control	ATCC 12744	No	25	0.56
9	ATCC 12744	Yes	30	0.40
Control	UV2-370	No	49	1.4
10	UV2-370	Yes	65	1.2

The tabulated results show that an isolate(strain) of each of the four species and its corresponding derived high ascorbic acid-producing strain grown under the glucose pulsing conditions of the invention produced enhanced yields of ascorbic acid, expressed as maximum ascorbic acid concentration in mg/liter, relative to growth under simple fed batch conditions, i.e., with no glucose addition after growth. The results also show that *C. pyrenoidosa* UTEX 1663 and its high L-AA producing mutant UV101-158, C. regularis UTEX 1808 and its high L-AA producing mutant UV5-280, and P. zopfii UTEX 1438 and its high L-AA producing mutant UV3-132 all provide enhanced specific formations of L-AA (mg/g cells) under the invention conditions, indicating improved utilization of the carbon source for ascorbic acid production relative to that obtained without glucoseaddition after the initial growth and glucose depletion stages.

It will be noted *C. pyrenoidosa* strain 343, in contrast to the other *C. pyrenoidosa* strains did not provide an increased ascorbic acid yield under either set of conditions; also that although each of the *Ankistrodesmus braunii* strains yielded a higher concentration of ascorbic acid under the invention conditions, neither one afforded improvement in the specific formation of the acid. It should be noted in this regard, however, that the particular set of conditions employed after the initial growth and glucose depletion stages had been optimized for C. pyrenoidosa strain UTEX 1663 with no attempt made to manipulate the conditions to enhance ascorbic acid production for either C. pyrenoidosa UTEX 343 or A. braunii ATCC 12744 or UV2-370. It is believed likely improved results may be obtained with these organisms as well.

EXAMPLE 11 (BEST MODE)

The procedure of Example 1 was followed except that (a) 5.6 mg ferrous sulfate, instead of 11.2 mg, was employed in the initial 0.6 L distilled water charge; (b) the nutrient solution consisted of:28 g glucose in 40 ml distilled water; 0.53 g trisodium citrate dihydrate plus 0.2 g magnesium sulfate in 20 ml;0.65 g each of monopotassium acid phosphate and disodium acid phosphate in 20 ml; and 4.7 ml of the trace metal solution plus 1 ml sulfuric acid in 15 ml; and (c) the actively growing culture of was strain UV 232-1 of Table I.

The temperature was raised to 35°C, agitation begun at 350 rpm, air passed through the medium at 0.2 liters/min (lpm), the pH adjusted to 6.9

with anhydrous ammonia (NH_3) added to the air flow, and strain UV 232-1 added to the medium. NH_3 was fed throughout the run as nutrient nitrogen source and pH controller. After 6.2 hours the air flow was increased to 0.4 lpm,the agitation to 400 rpm. At 11.8 hours the air flow was raised to 0.6 lpm, where it was held for the remainder of the run, and the agitation rate raised to 650 rpm. After 12.4 hours, 40 ml more of the glucose-containing nutrient solution was added to the fermentor, followed by 20 ml more at 24.4 hours and 15 ml more at 26.3 hours; in the meantime, theagitation rate was raised to 750 rpm at 22.8 hours, then adjusted to 900 rpm at 34.8 hours and maintained at that rate for the rest of th run.

The glucose content of the medium became depleted at 31.7 hours, at which time the cell density (C.D.) was 19.5 and the L-AA concentration was 322 mg/L. Glucose (2 ml of 10% solution) was fed to the fermentor at 41.7 hours and every 3 hours from 41.7 to 94.8 hours. Cell density and L-AA cocentration were followed with time as tabulated below along with calculated L-AA content of the biomass.

TABLE 5

Time hr	pH	C.D. g/l	L-AA mg/L	comments	L-AA
0	6.9	--			
6.2	6.9				
11.8	6.9		40ml glucose at 12.4 hrs, 20 at 24.4hrs, 15 at 26.3 hrs		
22.8					
31.7	6.9		glucose depleted		
34..8	7.0	19.5	322	*	
	16.5				
46.9	7.7	18.7	429		
	22.9				
53.6	7.8		528		
58.6	7.8	17.9	613		
	34.2				
70.8	7.9		694		

77.7	7.9	17.3	819
	47.3		
82.8	7.8		915
94.8	7.6	17.6	945
	53.7		

* 2 ml 10% glucose added at 41.7 hrs and every 3 hrs thereafter until 94.8 hrs.

The above procedure was repeated four more times, substantially as described. The percent L-AA averaged 5.2%of the dry weight of the biomass over the 5 runs.

Having thus described and exemplified the invention with a certain degree of particularity, it should be appreciated that the following claims are not to be so limited but are to be afforded a scope commensurate with the wording of each element of the claim and equivalents thereof.

Claims

1. An process for L-ascorbic acid production comprising the steps of:
(a) heterotrophically growing cells of a micro-organism capable of producing L-ascorbic acid heterotrophically in a growth promoting nutrient medium containing suitable a carbon source and dissolved oxygen, each in amounts sufficient for cell growth and L-ascorbic acid production,
(b) allowing the organism to grow in an initial stage to a high cell density accompanied by the formation of a first amount of the ascorbic acid and resulting in the substantially complete depletion of the carbon source,
(c) maintaining the cells in the substantially complete absence of the carbon source in the presence of dissolved oxygen until (i) cell growth substantially ceases and (ii) subsequent addition of carbon source in controlled amounts results in the formation of additional quantities of L-ascorbic acid with substantially no increase in cell density, and
(d) continuing the controlled carbon sourceaddition in the presence of oxygen until a desired increase in L-ascorbic acid production is attained with substantially no increase in the cell density.

2. The process of Claim 1 wherein the ratio of the total amount of L-ascorbic acid produced to the total cell density in step (d) is greater than such ratio in step (c) when cell growth has substantially ceased and the cell density has ceased to increase.

3. The process of Claim 1 wherein the ascorbic acid is recovered from the cells substantially free of cellular material.

4. The process of Claim 1 wherein the carbon source is present in non-repressing amounts.

5. The process of Claim 4 wherein the carbon source is a glucose source.

6. The process of Claim 5 wherein the glucose source is a saccharide or polysaccharide convertible to glucose in situ in the nutrient medium during the course of the fermentation.

7. The process of Claim 5 wherein the glucose source comprises glucose.

8. The process of Claim 1 wherein the microorganism is a green microalgae.

9. The process of Claim 8 wherein the microalgae is a species of the genus *Chlorella*.

10. The process of Claim 9 wherein the species is *pyrenoidosa*.

11. The process of Claim 10 wherein the organism is a strain of *C. pyrenoidosa* designated UV101-158, a mutagenized strain of *C. pyrenoidosa* UTEX 1663 and having A.T.C.C. accession No. 53170.

12. The *Chlorella pyrenoidosa* strain having A.T.C.C. accession No. 53170.

13. The *Chlorella pyrenoidosa* strain UV 232-1 descended from *C. pyrenoidosa* by UV irradiation.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/02429

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P17/04; C12N1/12; //(C12N1/12,C12R1:89)
(C12P17/04,C12R1:89)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12P ; C12R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0 207 763 (BIO-TECHNICAL RESOURCES, INC.) 7 January 1987 & US,A,5 001 059 (BIO-TECHNICAL RESOURCES, INC.) cited in the application see the whole document	1-12
A	CHEMICAL ABSTRACTS, vol. 98, no. 19, 9 May 1983, Columbus, Ohio, US; abstract no. 157610v, RENSTROEM B. ET AL 'Biosynthesis of L-ascorbic acid in Chlorella pyrenoidosa' page 246 ;column L ; see abstract & PLANT SCIENCE LETTERS vol. 28, no. 3, 1983, pages 299 - 305	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 JUNE 1993	[illegible] 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9302429
SA 71993

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 29/06/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0207763	07-01-87	US-A- 5001059	19-03-91
		JP-A- 62029994	07-02-87